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Differential scanning fluorimetry based assessments of the thermal and kinetic stability of peptide-MHC complexes

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Abstract

Measurements of thermal stability by circular dichroism (CD) spectroscopy have been widely used to assess the binding of peptides to MHC proteins, particularly within the structural immunology community. Although thermal stability assays offer advantages over other approaches such as IC₅₀ measurements, CD-based stability measurements are hindered by large sample requirements and low throughput. Here we demonstrate that an alternative approach based on differential scanning fluorimetry (DSF) yields results comparable to those based on CD for both class I and class II complexes. As they require much less sample, DSF-based measurements reduce demands on protein production strategies and are amenable for high throughput studies. DSF can thus not only replace CD as a means to assess peptide/MHC thermal stability, but can complement other peptide-MHC binding assays used in screening, epitope discovery, and vaccine design. Due to the physical process probed, DSF can also uncover complexities not observed with other techniques. Lastly, we show that DSF can also be used to assess peptide/MHC kinetic stability, allowing a single experimental setup to probe both binding equilibria and kinetics.

Keywords

MHC protein; binding affinity; thermal stability; kinetic stability; differential scanning fluorimetry

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1. Introduction

Quantifying the strength of the interactions between peptides and major histocompatibility complex (MHC) proteins is important in the identification of T cell epitopes and evaluating the consequences of naturally occurring or engineered peptide modifications. In many cases though, the instability of peptide-free “empty” MHC proteins (particularly for class I) has hindered direct measurements of MHC-peptide binding equilibria. To circumvent this complexity, a variety of experimental approaches have been developed that yield proxies for MHC-peptide binding affinities. Perhaps the most common is the use of competition experiments to report IC_{50} values relative to a labeled competitor (Ruppert et al., 1993). Other assays have been developed, including the use of pre-oxidized protein to reduce instability issues (Harndahl et al., 2009) and indirect assays that rely on antibody or T cell receptor detection of peptide/MHC complexes (Miles et al., 2011).

In addition to the approaches above, measurements of peptide/MHC thermal stability are commonly used to assess how well peptides interact with MHC proteins. Folded proteins exist in an equilibrium with their unfolded states, and due to the principle of coupled equilibria, ligand binding to a folded protein will increase protein thermodynamic stability. Due to this link, the presence of a bound ligand increases protein thermal stability, and the increase in a protein's T_m (formally defined as the temperature at which 50% of the protein is unfolded) correlates with binding affinity (Crothers, 1971; Brandts and Lin, 1990). Measurements of peptide/MHC thermal stability have been widely adopted by the structural immunology community, with circular dichroism (CD) spectroscopy being the most frequent mode of detection. In this use, CD monitors the loss of α -helical character in the peptide binding domain as the complex dissociates and unfolds, and early studies demonstrated close agreement between peptide/MHC thermal stability and robust affinity determinations by equilibrium dialysis (Morgan et al., 1997). Thermal stability measurements, whether by CD or other approaches (Schlundt et al., 2009; Schlundt et al., 2012) are advantageous in that they are not dependent on the identity or concentration of a competitor peptide. They can also be easily implemented, as they do not require peptide or protein modifications and use relatively simple instrumentation.

A drawback of CD-monitored thermal unfolding of peptide/MHC complexes is the limited signal-to-noise ratio and the large amount of sample required (typically hundreds of microliters of protein at concentrations of tens of micromolar). Although small relative to the significant amount of protein typically needed for structural studies, these requirements nevertheless reduce throughput and limit the use of thermal stability measurements in broader studies such as epitope discovery. Although small volume CD systems can reduce sample requirements, these suffer from low signal-to-noise, and medium-throughput CD instrumentation requiring complex liquid handling robotics has only recently been described (Fiedler et al., 2012).

Differential scanning fluorimetry (DSF; also referred to as ThermoFluor) provides an alternative to CD spectroscopy as a method to assess protein stability and the impact of ligand binding (Pantoliano et al., 2001; Matulis et al., 2005; Niesen et al., 2007). DSF takes advantage of small, environmentally-sensitive fluorescent molecules whose fluorescence is

enhanced when specifically bound to exposed hydrophobic surfaces such as those created by protein unfolding. Due to the high sensitivity of modern fluorimeters, DSF is well suited to low volume, rapid measurements on multiple samples. DSF has found numerous uses in characterizing protein stability and ligand binding, and has been recently used to assess the binding of peptides to class I MHC proteins (Gras et al., 2012; Koch et al., 2013a; Koch et al., 2013b; Hassan et al., 2015), as well as the impact of peptides and other molecules binding to class II proteins (Günther et al., 2010; Rupp et al., 2011; Clayton et al., 2014), including HLA-DM (Álvaro-Benito et al., 2015). Although early applications of DSF required customized fluorimeters, the technique is easily adaptable to fluorescence-equipped RT-PCR instruments common to many molecular biology laboratories. DSF is thus highly scalable, with one experimental run capable of assaying dozens of small-volume samples in plate format. Here we performed a comparison of DSF and CD spectroscopy for measuring the thermal stability of both class I and class II peptide/MHC complexes. We found DSF a straightforward, easily implementable approach that yields results equivalent to those obtained from CD spectroscopy while considerably reducing sample requirements.

Lastly, interest in peptide/MHC kinetic as opposed to thermodynamic stability has been growing. In some instances, kinetic stability may be a more biologically relevant parameter, as it more accurately reflects the situation of a MHC-presented peptide on a cell surface. Indeed, some reports indicate that kinetic stability is an improved indicator of antigenicity and immunodominance than binding affinity (Lazarski et al., 2005; Baumgartner et al., 2010; Harndahl et al., 2012). Various approaches have been used to measure peptide-MHC kinetic stability, including using fluorescent or radioactive peptides, surface plasmon resonance, and a recently developed scintillation proximity assay (Gakamsky et al., 1996; Gakamsky et al., 2000; Binz et al., 2003; Baxter et al., 2004; Harndahl et al., 2011; Miles et al., 2011). We show here that for class I peptide/MHC complexes, DSF readily lends itself to direct measurements of peptide dissociation without the need for protein or peptide labeling.

2. Materials and Methods

2.1 Peptides and proteins

Peptides were synthesized commercially (CHI Scientific or 21st Century Biochemicals). For class I protein, recombinant HLA-A*0201 heavy chain and β_2 -microglobulin were expressed as inclusion bodies in *Escherichia coli* (Garboczi et al., 1992). MHC folding and assembly from inclusion bodies was performed according to standard procedures (Pierce et al., 2014). Protein was purified using ion exchange followed by size-exclusion chromatography. For class II protein, soluble extracellular domains of HLA-DR1 (DRA*0101/DRB*010101) were expressed in *Drosophila* S2 cells and purified by immunoaffinity chromatography as previously described (Sloan et al., 1995).

2.2 Differential scanning fluorimetry

Differential scanning fluorimetry was performed using Applied Biosystems StepOnePlus (for class I complexes) or Bio-Rad C1000 Thermal Cycler (for class II complexes) RT-PCR instruments with the excitation and emission wavelengths set to 587 and 607 nm, respectively. For class I complexes, solution volumes were 20 μ L in 96-well plates. Assay

buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, with 0.005% surfactant P20 added to prevent protein adherence to the plate. For class II complexes, solution volumes were 25 μ L in 96-well plates. Assay buffer was 100 mM sodium citrate (pH 5.5), 50 mM NaCl, 5 mM EDTA, 0.1% NaN₃, 1 mM DTT, 1 mM PMSF, and 0.1% octylglucoside. The different solution conditions for class I and class II complexes were based on optimal conditions for preparation and storage of the different complexes, and are conventional buffers for the two systems (Khan et al., 2000; Yin et al., 2015). For class II complexes, the low pH is believed to approximate conditions for endosomal loading. Pilot studies of class I complexes under the conditions used to assess class II resulted in substantial reductions in apparent T_m .

For thermal stability measurements, the temperature scan rate was fixed at 1 $^{\circ}$ C/min unless otherwise indicated. Protein and SYPRO orange (Invitrogen) concentrations were varied as described. The temperature range spanned 20 $^{\circ}$ C to 95 $^{\circ}$ C. Data analysis was performed in OriginPro 7 or 9. Apparent T_m values were first determined by identifying the point at which the transition was 50% complete. For a more rigorous analysis, the temperature derivative of the melting curve was computed. The resulting derivative curve was processed with the peak fitting algorithm in OriginPro, applying a sigmoidal baseline and fitting the peak to determine the T_m and its standard error. A bi-Gaussian function, commonly used in spectroscopy and chromatography, was used for peak fitting as the peaks were usually noticeably skewed, presumably due to contributions from irreversible aggregation that occurs co-incident with unfolding. When multiple transitions were observed, the data were subject to the automated multiple peak fitting process in OriginPro, using default options except that bi-Gaussian functions were used. This proved successful in identifying the major transitions and their associated T_m and standard error as shown in **Figs. 1C and 3A**. For kinetic measurements, 10 μ M of protein was incubated in the instrument at a constant temperature of 37 $^{\circ}$ C with 10X SYPRO orange, with fluorescence measured every five minutes. Kinetic data were fit to a biexponential function. As performed previously (Binz et al., 2003), the slowest rate constant was attributed to peptide dissociation from the class I MHC heterotrimer and used to determine half-lives.

2.3 Circular dichroism spectroscopy

CD spectroscopy was performed as previously described (Khan et al., 2000; Borbulevych et al., 2010; Borbulevych et al., 2011; Ekeruche-Makinde et al., 2012), using a Jasco J815 instrument. Temperature was increased from 10 $^{\circ}$ C to 100 $^{\circ}$ C at an increment of 1 $^{\circ}$ C/min, monitoring a wavelength of 218 nm. Protein concentrations were between 5 μ M and 10 μ M in 20 mM phosphate (pH 7.4), 75 mM NaCl. Data analysis was performed in Kaleidagraph or OriginPro. Data in the transition region were differentiated or fit to a polynomial, and apparent T_m values were determined as the point in which the transition was 50% complete.

2.4 IC₅₀ measurements

IC₅₀ measurements for peptide binding to HLA-DR1 were determined using a fluorescence polarization assay, using Alexa488-labeled HA₃₀₆₋₃₁₈ as the indicator peptide as previously described (Yin et al., 2012; Yin and Stern, 2014).

3. Results

3.1 Implementation and optimization of DSF for peptide/MHC stability

Differential scanning fluorimetry was implemented utilizing the environmentally-sensitive fluorescent molecule SYPRO orange. SYPRO orange is minimally fluorescent in solution, but experiences a >400% enhancement of fluorescence in the presence of unfolded protein by binding exposed hydrophobic regions (Niesen et al., 2007). For class I complexes, we utilized a StepOnePlus RT-PCR instrument, using a solution volume of 20 μ L in 96 well plates. We first explored the thermal stability of the tyrosinase peptide (TYR, sequence YMDGTMSQV) bound to the class I MHC protein HLA-A*0201 (A2). With a protein concentration of 10 μ M, 10X SYPRO orange (SYPRO orange is provided as a 5000X stock solution; the actual concentration, chemical composition, and molecular weight are withheld by manufacturer), and a scan rate of 1 $^{\circ}$ C/min we observed a clear fluorescence response similar to that seen in DSF studies of other protein-ligand interactions, including peptide binding to MHC (e.g., (Matulis et al., 2005; Niesen et al., 2007; Clayton et al., 2014; Hassan et al., 2015). The progress curve was sigmoidal over the temperature range of 30 $^{\circ}$ C to 70 $^{\circ}$ C, demonstrative of cooperative thermal unfolding (**Fig. 1A**). Unlike thermal denaturation by CD, there was virtually no scatter in the data, eliminating the need to fit the data to a polynomial in order to determine the midpoint of the curve. For the TYR/A2 complex, the midpoint occurred at 67 $^{\circ}$ C. We refer to this midpoint as the “apparent T_m ” (sometimes simplified to just T_m ; we use the word “apparent” as the complexity of peptide/MHC systems hinders rigorous thermodynamic analyses as discussed below).

To explore sensitivity, we assessed unfolding of the TYR/A2 complex as a function of concentration, reducing concentration in 2 μ M steps with SYPRO orange concentration held constant at 10X. Although fluorescence intensity dropped with concentration, signal-to-noise remained high, with a clear sigmoidal curve visible at a concentration as low as 2 μ M (**Fig. 1A**). As judged by the midpoint of the curve, the apparent T_m showed no variation with concentration over this range. We also varied the SYPRO orange concentration from 5X to 20X with no systematic variation in T_m , but obtained the clearest signal with 10X.

3.2 T_m values determined by DSF and CD spectroscopy are comparable for both class I and class II complexes

We next examined a large number of different peptide/A2 complexes. These complexes included peptides with a wide range of A2 binding affinities, from weak (MART-1₂₇₋₃₅/A2) to strong (HTLV-1 Tax₁₁₋₁₉/A2). As anticipated, we observed a wide range of apparent T_m values, detecting not only large differences between peptides and significant consequences of anchor residue modification, but also subtler effects resulting from more conservative peptide modifications (**Table 1**).

We also used an alternative approach for determining T_m values from the data. Quantitative analyses of sigmoidal transitions can be performed (Niesen et al., 2007), but accuracy requires accounting for pre- and post-transition baselines (Pace, 1990). We took a simpler approach derived from analysis of differential scanning calorimetry data and utilized the maximum of the first derivative of the sigmoidal curve rather than the midpoint of the

transition, determined by subjecting the transition to a quantitative peak analysis (**Fig. 1B**). This approach is advantageous as it can be more easily implemented and provides an objective means of determining error in the T_m . In all cases, the T_m values determined via assessing 50% of the sigmoidal transition and the midpoint of the first derivative were nearly identical. To assess reproducibility, we repeated the measurement of the MART-1_{26(2L)-35}/HLA-A2 complex three times using separate samples. The data were in very close agreement: the standard deviation of the T_m was ± 0.2 °C, less than the fitting error determined from the quantitative peak analysis.

We next assessed the applicability of DSF for determining the stability of class II peptide/MHC complexes using the well-characterized complex of HLA-DR1 (DR1) bound to the influenza HA peptide (PKYVKQNTLKLAT) (Sato et al., 2000a). We observed a clear transition with a high T_m of 83 °C, as shown in **Fig. 1C**. As DR1 can be obtained by expression in insect cells as empty protein (free of associated peptides) (Yin et al., 2015), we were also able to assess empty DR1, which unfolded with a T_m of 69 °C. As with the class I complexes, the stability of peptide/DR1 complexes varied with peptide (**Table 1**).

The thermal stabilities of six of the peptide/A2 complexes and all four of the peptide/DR1 complexes in **Table 1** have been previously determined using CD spectroscopy (Khan et al., 2000; Sato et al., 2000b; Borbulevych et al., 2010; Borbulevych et al., 2011; Ekeruche-Makinde et al., 2012). We utilized CD to determine the stabilities of the remainder, using our previously established methods. The values determined by fluorimetry were in excellent agreement with those determined by CD: the CD and DSF values were highly correlated (correlation coefficient (R) of 0.97), and there were no systematic differences between the DSF and CD data (**Fig. 1D**). The close agreement between the DSF and CD is all the more impressive given that the CD data were collected for class I and class II systems in different laboratories over a period of several years.

3.3 Irreversibility leads to a scan rate dependence of peptide/A2 thermal unfolding

A complexity of peptide/MHC thermal stability measurements is that, as assessed by CD spectroscopy, thermal unfolding is irreversible due to aggregation (although the extent varies with class and allele). Irreversibility limits more detailed interpretation of T_m values beyond serving as a proxy for affinity and hinders determination of thermodynamic parameters. To examine if the greater sensitivity of DSF could better detect reversibility, we cooled peptide/A2 samples to 25 °C after monitoring unfolding and repeated the measurement after equilibration at 25 °C for 10 minutes. Despite the greater sensitivity of fluorimetry compared to CD, no clear indications of reversibility were observed, as shown in **Fig. 1E** for the SL9/HLA-A2 complex.

We next assessed the influence of scan rate on the apparent T_m . Scan rate is a key parameter in thermal unfolding experiments as heat transfer can be rate limiting. In cases where irreversible thermal unfolding occurs, scan rate can also influence side reactions such as aggregation. A scan rate dependence is another tool for demonstrating irreversible thermal unfolding (Sanchez-Ruiz et al., 1988; Ibarra-Molero et al., 2016). Using CD, a slight scan rate dependence has been shown for thermal denaturation of DR1 (Zarutskie et al., 1999).

Exploring different peptide/A2 complexes and altering scan rate from 0.5 °C/min to 2 °C/min, we observed regular variations in T_m , with lower scan rates yielding lower T_m values, as shown in **Fig. 1F** for the anchor-modified MART-1_{26(2L)-35}/HLA-A2 complex. Given this variation, we maintained the 1°C/min scan rate for the remainder of the study as it maintains consistency with prior CD measurements.

3.4 T_m values determined by DSF correlate with predicted and measured IC_{50} values

We next examined the agreement between DSF-determined T_m values and predicted and measured peptide binding affinities (IC_{50} values). Using the NetMHC 3.4 and NetMHCII 2.2 servers (Lundegaard et al., 2008; Nielsen and Lund, 2009), we predicted the affinities of each of the peptide/MHC complexes in **Table 1** (excluding the YRAL peptide, which at four amino acids is beneath the length threshold for NetMHCII). In aggregate the results were reasonably well correlated ($R = 0.72$; **Fig. 2A**). Evaluating the class I and class II data separately yielded improved correlations: 0.84 for class I and 0.99 for class II (although the agreement for the class II data seems remarkable, the significance of this is debatable given the small number of data points).

We then compared the DSF-determined T_m values with experimentally-determined IC_{50} values. As peptide exchange with class I MHC proteins is complicated by slow kinetics and protein aggregation, we restricted this comparison to a class II system. We recently published IC_{50} values for the HLA-A2₁₀₄₋₁₁₇ peptide and five variants binding to DR1 (Yin et al., 2014). Values for an additional 14 peptides were determined here using our previously established methods (Supplemental Table 1). As shown in **Fig. 2B**, the IC_{50} data were well correlated with the T_m values determined by DSF ($R = 0.85$), confirming the relationship between T_m and peptide-MHC binding affinity.

3.5 DSF detects β_2 -microglobulin dissociation from low stability complexes

In the analysis of some class I complexes, we observed multiple transitions in the thermal melting curves. This was most reproducible in the lowest stability complexes we studied, those with the HUD (sequence LGYGfVNYI) and MART-1₂₇₋₃₅ (sequence AAGIGILTV) nonamer peptides bound to HLA-A2 (**Fig. 3A**). Although the DSF-determined T_m values for the major transitions in these cases were close to T_m values determined by CD (**Table 1**), the presence of a second transition was surprising, particularly as multiple transitions have not been seen by CD for these peptides. The T_m of the second transition was 62 - 63 °C.

A limitation of CD-based thermal measurements of class I peptide/MHC stability is the inability to detect unfolding of β_2m due to its lack of α -helical character. As DSF probes a different physical process than CD (exposure of hydrophobic surfaces vs. helix content), we asked whether the second peak observed in the sample with the HUD and MART-1₂₇₋₃₅ peptides reflected melting of free β_2m . DSF of β_2m alone showed a peak with a T_m of 63 °C (**Fig. 3A,B**). As all samples were carefully purified, the β_2m transition is not likely attributable to excess β_2m in the sample. We thus attribute the additional transition in the HUD and MART-1₂₇₋₃₅ samples to the presence of dissociated β_2m , resulting from low temperature-induced breakdown of the complex. As multiple transitions are not detected in the more stable complexes, we presume that with higher affinity peptides dissociation and

unfolding proceeds in a more cooperative manner (further support for this interpretation is found in Fig. 3A, in which the β_2m peak is more clearly defined in the lowest stability complex). This conclusion is consistent with studies of the denaturation of mouse H-2D^b and H-2K^b complexes, which followed different paths for low and high affinity peptides (Saini et al., 2013).

3.6 SF-based measurements of class I peptide/MHC kinetic stability

We last explored the ability of DSF to measure the kinetic stability of class I peptide/MHC complexes, taking advantage of the rapid aggregation of class I heavy chains that occurs after peptide dissociation (Gakamsky et al., 1996; Gakamsky et al., 1999). We examined two MART-1 peptides: the 27-35 nonamer (AAGIGILTV) and the 26-35 decamer (EAAGIGILTV), in addition to the corresponding anchor-modified variants in which the alanines at peptide position two are replaced with leucine (ALGIGILTV and ELAAGIGILTV). Each sample was incubated in the RT-PCR instrument at a constant temperature of 37 °C for approximately 10 hours at a protein concentration of 10 μ M with 10X SYPRO orange. Fluorescence intensity was read every five minutes. All four samples showed exponential increases in fluorescence intensity (Fig. 4), indicative of peptide dissociation and subsequent protein aggregation. Supporting this interpretation, the progress curves for both anchor-modified peptides were slower.

Consistent with our previous studies of peptide dissociation from HLA-A2 using fluorescence anisotropy (Binz et al., 2003; Baxter et al., 2004), the dissociation data were fit best by a biphasic exponential function. As in our previous work we attribute the slowest phase to peptide dissociation from the class I heterotrimer. Accordingly, anchor-modification of the MART-1₂₇₋₃₅ nonamer increases the half-life of the complex from 4.4 to 6.9 hours. Anchor-modification of the MART-1₂₆₋₃₅ decamer increases the half-life from 3.2 to 16.9 hours. We note that for the MART-1₂₆₋₃₅ decamer peptides, the half-lives are nearly 2-fold shorter than those determined previously using a surface plasmon resonance-based assay (Cole et al., 2010). The most likely reason for this is the previous studies were performed at a lower temperature, which yields slower kinetics (25 °C vs. 37 °C; our use of the higher, more physiologically relevant temperature was necessitated by the need to promote the aggregation required for SYPRO orange binding and fluorescence). However, the 5.3-fold *change* in half-life for the anchor-modified MART-1 decamer reported here is exactly the same as that reported previously, indicating that DSF provides a convenient alternative for assessing class I peptide/MHC kinetic stability.

4. Discussion and Conclusions

Over the past two and a half decades, multiple approaches for quantifying the interactions of peptides with MHC proteins have been described. Measurements of thermal stability by circular dichroism spectroscopy have been widely adopted for this purpose by the structural immunology community, as well as investigators studying the physical properties of peptide/MHC complexes. Downsides to CD-based thermal stability measurements are the large sample requirements and single-sample throughput. Here we demonstrate that an alternative approach based on differential scanning fluorimetry yields results comparable to

those based on CD. We emphasize that, because of the complexity of peptide/MHC complexes and their tendency to denature irreversibly, the analysis of thermal stability measurements, whether by CD or DSF, does not allow for straightforward extraction of rigorous thermodynamic parameters. A consequence is that comparative experiments must be collected under well controlled experimental conditions maintained across samples. In this way, and despite their underlying complexity, the apparent T_m values allow for a rapid and straightforward assessment of how strongly peptides interact with MHC proteins.

Additionally, the same physical process that allows for T_m determinations also readily lends itself to measurements of kinetic stability. Both types of measurements can be performed in widely available RT-PCR instruments, permitting the simultaneous analysis of multiple samples. The greater sensitivity of fluorescence translates into low sample requirements, illuminating the potential for DSF to aid not only physical and structural studies of select antigens, but also experiments that depend on the rapid assessment of many samples, such as studies in epitope discovery, vaccine design, and immune evasion. In principle, protein production strategies for both class I and class II MHC could be scaled down to facilitate DSF's integration into a true high throughput pipeline.

DSF also has potential to identify characteristics of MHC-peptide interactions not visible via other techniques. An example here is the detection of dissociated β_2m from low-stability class I complexes. In principle, the analysis could detect additional complexities, such as partial/multi-step dissociation, register-shifting, or multi-mode binding. Partial dissociation has been implicated in peptide immunogenicity (Duan et al., 2014) and in facilitating optimal T cell receptor binding (Madura et al., 2015). Register shifting or multi-mode binding has been seen in peptides that bind both class I and class II MHC proteins (Borbulevych et al., 2007; Landais et al., 2009; Günther et al., 2010). The binding of ANS, which like SYPRO orange interacts with exposed hydrophobic surfaces, has been used to examine pH dependent conformational changes in class II MHC proteins (Boniface et al., 1996; Runnels et al., 1996). Insight into these complexities would not be available from other methods to study MHC-peptide binding such as IC_{50} measurements or CD spectroscopy. This potential to uncover complexities in how peptides interact with MHC proteins, combined with its enhanced sensitivity, lower sample requirements, and capacity for high throughput assessments of both thermal and kinetic stability make DSF an appealing tool for assessing the interactions of peptides and other ligands with MHC proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DSF	differential scanning fluorimetry
T_m	melting temperature
CD	circular dichroism

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Highlights

- The thermal stability of peptide/MHC complexes correlates with peptide affinity
- Differential scanning fluorimetry permits rapid assessment of peptide/MHC stability
- Substantially lower volume requirements of DSF provides for high throughput
- DSF also allows measurements of peptide/MHC kinetic stability / dissociation rates
- Complexities not detectable with other binding assays can be observed with DSF

Figure 1.

Quantitative analysis of differential scanning fluorimetry in assessing class I and class II MHC/peptide stability. **A)** Progress curves for different concentrations of the class I TYR/A2 complex. Overall fluorescent intensity varied with concentration, but the midpoint of the transition was a constant 67 $^{\circ}\text{C}$, as indicated by the vertical dashed line. **B)** The first derivative of the progress curves provides a means to robustly determine T_m values, as shown for the TYR/A2 complex at 2 μM . Black dots indicate the first derivative data; the red line is a baseline-corrected bi-Gaussian peak fit. **C)** First-derivative analysis and associated fit of the data for the class II HA/DR1 complex (circles) and 'empty' DR1 (triangles). **D)** peptide/MHC T_m values determined by DSF correlate with those determined by CD spectroscopy. Black circles are class I complexes; blue triangles are class II. The correlation coefficient (R) for the linear fit to the data (red line) is 0.98. **E)** peptide/A2 denaturation remains irreversible when studied by DSF. The black curve shows the DSF scan of a fresh sample of the SL9 peptide bound to A2 with a T_m of 59 $^{\circ}\text{C}$. The purple line shows a rescan of the same sample after cooling to 25 $^{\circ}\text{C}$. **F)** Thermal denaturation of peptide/A2 complexes show a scan rate dependence, as indicated here for the MART1_{26(2L)}-35 peptide bound to A2.

Figure 2.

T_m values determined by DSF correlate with predicted and measured IC_{50} values. **A)** Comparison of DSF-determined T_m values and IC_{50} values predicted by NetMHC and NetMHCII. Black circles are class I complexes; blue triangles are class II. The solid red line is a linear fit to the class I and class II data together. The upper dashed line is a linear fit to the class II data alone, and the lower dashed line is a linear fit to the class I data alone. Correlation coefficients for all three fits are indicated. **B)** Comparison of DSF-determined T_m values and experimental IC_{50} values for peptide/DR1 complexes. The red line is a linear fit to the data. The correlation coefficient is 0.85.

Figure 3.

DSF detects separate β_2m unfolding in low stability class I MHC complexes. **A)** Derivative analyses of the progress curves for the MART-1₂₇₋₃₅ and HUD complexes with A2. A separate peak is seen for both with a T_m at or near 63 °C. The pink dashed line shows the fit from an analysis of β_2m alone superimposed on the MART-1 and HUD data. **B)** DSF analysis of β_2m alone shows a single transition with a T_m of 63 °C. The fitted curve from this analysis is shown as the pink dashed line in panel A. The reduced intensity of the β_2m alone sample in panel B compared to the pMHC samples in panel A is attributable to a lower concentration and the smaller size of β_2m , resulting in less binding SYPRO orange.

Figure 4.

Differential scanning fluorimetry can be used to assess peptide dissociation kinetics and thus the kinetic stability of class I MHC complexes. **A)** Dissociation of the native MART-1₂₇₋₃₅ nonamer (circles) and anchor-modified variant (triangles) from HLA-A2 at 37 °C. **B)** Dissociation of the native MART-1₂₆₋₃₅ decamer (circles) and anchor-modified variant (triangles) from HLA-A2 at 37 °C. For both panels, red lines indicate biexponential fits to the data. The slowest time constant was used to report complex half-lives, indicated in the inset as $t_{1/2}$. Half-lives for the faster time constants in hours are 0.4 for AAGIGILTV, 0.5 hours for ALGIGILTV, 0.3 hours for EAAGIGILTV, and 1.1 hours for ELAGIGILTV.

Table 1

Comparison of peptide/MHC apparent T_m values determined by differential scanning fluorimetry and circular dichroism.

Peptide name	Sequence	T_m by DSF (°C)	T_m by CD (°C)
<i>Class I peptide/MHC complexes</i>			
MART-1 ₂₇₋₃₅	AAGIGILTV	40.8 (± 0.1)	37
MART-1 _{27(2L)-35}	ALGIGILTV	59.2 (± 0.2)	51
MART-1 ₂₆₋₃₅	EAAGIGILTV	45.7 (± 0.3)	39
MART-1 _{26(2L)-35}	ELAGIGILTV	59.4 (± 0.3)	55
TAX	LLFGYPVYV	66.3 (± 0.1)	65
LLE	LLEEMFLTV	54.0 (± 0.6)	52
gp100 _{209(2M)-217}	IMDQVPFSV	62.2 (± 0.5)	60
HUD	LGYGFVNYI	44.8 (± 0.3)	40
HUD _{87(2L,9V)-95}	LLYGFVNYV	65.1 (± 0.5)	63
NS3 ₁₄₀₆₋₁₄₁₅	KLVALGINAV	64.0 (± 0.6)	64
NS3 _{1406(4T)-1415}	KLVTLGINAV	63.0 (± 0.1)	64
NS3 _{1406(7N)-1415}	KLVALGNNAV	55.8 (± 0.2)	59
NS3 _{1406(3S,4G,7L)-1415}	KLSGLGLNAV	61.1 (± 0.1)	61
WT1	RMFPNAPYL	56.3 (± 0.3)	54
TYR	YMDGTMSQV	67.3 (± 0.1)	65
TYR (T5F)	YMDGFMSQV	61.4 (± 0.3)	63
<i>Class II peptide/MHC complexes</i>			
HA	PKYVKQNTLKLAT	82.1 (± 0.1)	84
FRR-HA-F308A	PRAVKQNTLRLAT	68.9 (± 0.1)	76
Min4	YRAL	67.8 (± 0.1)	71
Yak	AAYAAAAAKAAA	85.5 (± 0.1)	89